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INTRODUCTION

c-Myc is a bHLH-ZIP transcription factor that regulates the expression of a large number of target genes, which collectively promote transformation (1,2). The active form of c-Myc exists as a heterodimer with another bHLH-ZIP protein, Max (3,4). This interaction, along with c-Myc-Max sequence-specific DNA binding ability (3,4) is necessary for all of c-Myc's biological properties, including transformation.

c-Myc and Max are members of a group of interacting bHLH-ZIP proteins collectively referred to as the "Myc network" (3,4). Opposing the effects of c-Myc-Max heterodimers are four additional, closely related members termed the "Mad" family (Mad1, Mxi1, Mad3, and Mad4) and several less related proteins Mnt, Mga, and Mlx.

Positive regulation of target genes is a result of DNA binding by c-Myc-Max. Consensus sites, termed "E-boxes" (CAC/TGTG), are found in virtually all positively regulated c-Myc target genes (3,4). However, c-Myc-Max binding to these sites and activation of the adjacent gene(s) is also dependent upon other factors, including the affinity of the sites for the heterodimers, the levels of c-Myc, competition with other E-box binding factors, and the cell type (1,2).

In addition to Mad-dependent regulation, c-Myc also negatively regulates a separate subset of genes in an E-box- and Mad-independent manner. The means by which this occurs is somewhat obscure, although the most well-characterized example involves the interaction of c-Myc-Max with Miz1, a ubiquitously expressed POZ domain-zinc finger protein. This results in the transcriptional silencing of Miz1-dependent promoters (5). Similar forms of control may exist for other transcription factors such as YY1 and Sp1 (6,7). As in the case for c-Myc-dependent activation, repression also appears to be Max-dependent (6,7).

At first glance, inhibition of a ubiquitously expressed protein such as c-Myc could be dismissed as being unacceptably toxic. However, while it is true that all proliferating cells express c-Myc, quiescent cells do not. Thus, toxicity resulting from c-Myc inhibition should be mainly confined to highly proliferative compartments (e.g. bone marrow and GI tract) and thus be no more severe than that seen with more standard therapies. Moreover, considerable evidence now indicates that continuous c-Myc expression is necessary to maintain the transformed state and that even its transient c-Myc inhibition may be sufficient to promote tumor regression (8,9). The compounds generated in the current proposal should therefore be useful in testing these ideas.

Using a c-Myc-Max yeast-based 2 hybrid assay, we originally screened >10,000 low molecular weight compound and identified 7 that prevented/inhibited the c-Myc-Max interaction (10). We showed these compounds to be highly specific for c-Myc-Max, and to profoundly affect the growth rates of c-Myc over-expressing cells but not of c-Myc-/- cells. Unfortunately, these compounds were active only in the 20-50 mM range and could not be expected to be useful as clinically relevant therapeutics. In order to obtain more relevant compounds, we felt it necessary to address several important questions regarding these compounds:

1. How precisely did they affect the interaction between c-Myc and Ma

2. Could we obtain more potent analogs/

3. Could structural models of these compounds in association with their cognate binding sites be obtained that would aid in the creation of more potent analogs.

ORIGINAL SPECIFIC AIMS OF THE ORIGINAL APPLICATION

***Task 1*: To demonstrate that each of these compounds either prevents or disrupt c-Myc-Max heterodimerization.**

***Task 2*: To conduct a series of *in vivo* studies aimed as determining whether these compounds can be effectively employed to treat c-Myc overexpressing tumors.**

***Task 3*: To determine whether any of the compounds can be utilized in combination as a means of reducing toxicity and potentiating the antineoplastic effect.**

***Task 4*: To employ computerized "data mining" techniques to identify the structural properties of these compounds which impart their effectiveness.**

ACCOMPLISHMENTS OVER THE ENTIRE COURSE OF THE APPLICATION

***Task 1*: To demonstrate that each of these compounds either prevents or disrupt c-Myc-Max heterodimerization.**

As summarized in our 2007 progress report, we used recombinant versions of the c-Myc and Max bHLH-ZIP domains to demonstrate that three of the original parental compounds, namely 10058-F4, 10074-G5 and 10074-A4 (10) bound to monomeric c-Myc but not to monomeric or dimeric Max. These binding studies utilized fluorescence polarization-based measurements, which took advantage of the fact that the first two compounds were fluorescence and readily quantifiable. Recombinant deletion mutants of the c-Myc bHLH-ZIP domain as well as a series of >100 random point mutations were then used in similar types of experiments to map the actual binding sites of the three compounds. Based on these studies, we were able to show that each compound bound to a unique site within the c-Myc bHLH-ZIP domain (11 and submitted for publication). Interestingly, the other 4 members of the original 7 parental compounds bound to these same sites as well. These finding were confirmed with circular dichroism (CD) experiments showing that synthetic peptides containing the inferred binding sites also bound the compounds with affinities comparable to those of the longer deletion mutants (11 and submitted for publication).

The remaining compound, 10074-A4 was not fluorescent. However, we took advantage of the fact that circular dichroism (CD) could be shown to be an extremely effective alternative means of quantifying binding these compounds. Using CD and the above-mentioned deletion proteins, we showed that 10074-A4 bound at a site between that of 10058-F4 and 10074-G5 (not shown)

In order to confirm these studies, we again employed CD but this time utilized synthetic peptides or a very small recombinant peptide that spanned the putative binding sites of these compounds. As shown in Fig. 1, we were able to confirm these binding sites by showing alterations in the CD spectra in all three cases.

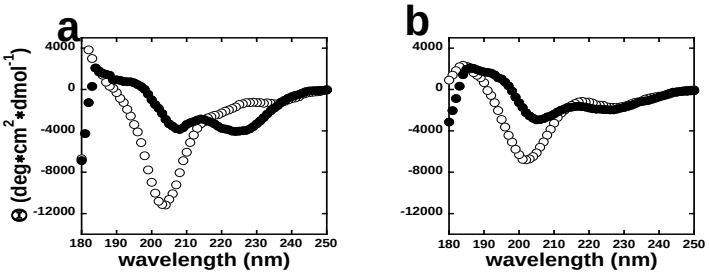
***Task 2*: To conduct a series of *in vivo* studies aimed as determining whether these compounds can be effectively employed to treat c-Myc overexpressing tumors.**

We have tested one of the original parental compounds, 10058-F4 in an in vivo animal model (12). We evaluated the pharmacokinetics, pharmacodynamics and efficacy of 10058-F4 against two androgen independent prostate cancer cell lines (PC3 and DU145) which express high levels of c-Myc and which are dependent on the oncoprotein for their growth and/or viability (13). We summarize the data here and refer the reviewer to our recently published manuscript Ref. 12 for a more in-depth description and analysis of our studies and the actual data.

A. Preliminary in vitro efficacy: IC_{50}'s of 10058-F4 against PC3 and DU145 prostate cancer cell lines determined at 72 h after a single addition were estimated using the Hill equation and were

respectively, 113 ± 30 μM and 88 ± 20μM. These concentrations are within the range of those previously reported for the inhibition of Rat1a-c-Myc fibroblasts (10).

B. In vivo efficacy. Treatment of SCID mice bearing DU145 or PC3 xenografts with either 30 or 20 mg/kg 10058-F4 on a qd x 5 schedule for two weeks did not significantly inhibit tumor growth (Ref. 12, Fig. 1). The maximum mean %TC (% treated tumor/control tumor volume) for the mice

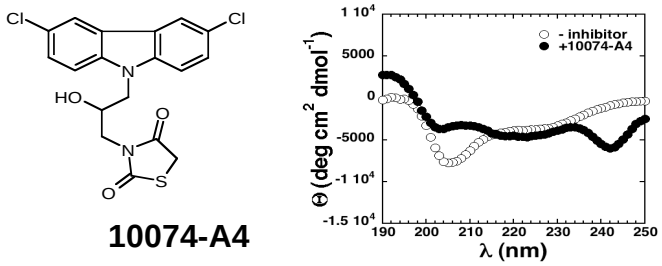


c

10058-F4-binding peptide: Y_{402} ILSVQAEEQK$_{412}$

10074-G5-binding peptide: E_{363}RQRRNELKRSFFALRDQI$_{381}$

d **e**



f 10074-A4-binding peptide: E_{370}LKRSFFALRDQIPELENNEKAPKVVILKKATAYILSVQAE$_{409}$

Fig. 1. Circular dichroism (CD) study of 10058-F4 (a) and 10074-G5 (b) bound to synthetic peptides corresponding to their deduced binding sites on the c-Myc bHLH-ZIP domain. The far UV CD spectrum of each peptide alone in buffer or the peptide plus the inhibitor in buffer with <5% ethanol (DMSO interferes at these wavelengths) were collected at 25 C and are presented as the average of three independent trials. Peptide and inhibitor were each present at 20 uM concentration. (a) Myc 402-412 alone (open circles) and $Myc_{402-412}$ with 10058-F4 (b)) $Myc_{363-381}$ alone (open circles) and $Myc_{363-381}$ with 10074-G5 (closed circles). (c). Sequences of the synthetic peptides used in the above CD studies. (d) Structure of the non-fluorescent parental Myc compound 10074-A4. The homogeneously pure recombinant deletion protein $Myc_{370-409}$ was used in CD studies as shown in e. (f). The binding site for 10074-A4

bearing PC3 xenografts treated with 30 mg/kg and 20 mg/kg 10058-F4 were 72.9 % and 72.3 % on day 18, respectively. In contrast, intravenous treatment with docetaxel on a q7d x 2 schedule resulted in significant tumor growth inhibition and a maximum mean % T/C of 20.2% on day 18. Similar responses were observed in mice bearing DU145 xenografts (Ref. 12, Fig. 1) Although mice bearing PC3 androgen-independent xenografts lost up to 18% body weight during the course of the study, this was observed in all groups and appeared to be related to the tumor xenograft. No significant decreases in body weight were observed in the mice bearing DU145 xenografts during the course of treatment.

C. Plasma Pharmacokinetics. The plasma concentration versus time curve of 10058-F4 following a single i.v. dose of 20 mg/Kg is shown in Ref. 12, Fig. 2. Peak plasma concentrations at 5 min in

mice bearing DU145 xenografts or PC3 xenografts were 368.5 and 272.7 μM, respectively. Plasma concentrations then decreased rapidly and were undetectable by 360 min. A two compartmental model best described these data.. The terminal half-life of 10058-F4 was approximately 1 h and the volume of distribution was small (>200 ml/Kg). Clearance was rapid and due primarily to the short life of of 10058-F4, with multiple metabolic peaks being observed in plasma and tissues (Ref. 12 Figs. 3-5)

D. Urinary excretion. <0.1% of 10058-F4 was detected as unchanged compound in the urine of mice bearing DU145 tumors, with 60% occurring within the first 24 hr.

E. Tissue concentrations. As shown in Ref. 12 Table 2, 10058-F4 distributed rapidly to all tissues following i.v administration, with peak concentrations detected in liver, kidney, lung, spleen, heart and skeletal muscle by 5 min, the earliest time sampled. In mice bearing DU145 tumors, 10058-F4 concentrations in fat and brain peaked slightly later, at 15 min and 10 min, respectively. The peak concentration of 10058-F4 in tumors was much lower than in other tissues. Concentrations in both DU145 and PC3 xenografts were relatively constant between 5 and 15 min, then decreased, and were not detected beyond 6 h. The peak concentration in either tumor was only about 5% of the peak plasma concentration. The disparity between plasma and tumor 1058-F4 levels may at least partly reflect the abnormal tumor vasculature; this, plus the short half-life of 10058-F4, likely explain the relatively poor response of the tumors.

F. Metabolite analysis. 10058-F4 metabolites were characterized by LC/MS in plasma, liver, and kidney from a mouse administered 20 mg/Kg 10058-F4 30 min prior to sacrifice. In addition to unmodified 10058-F4, at least 8 potential metabolite peaks were detected. As shown in Ref. 12, Fig. 5, these had mass to charge ratios of 232, 248, 250, 264, 266, 280, 282, and 298. The proposed structures of these metabolites are shown in Ref.12, Fig. 5.

G. 10058-F4 does not alter tumor c-Myc levels: Tumor c-Myc levels were analyzed by Western blot at 0, 2,4, and 24h after administration of 10058-F4 at 20 mg/kg to SCID mice bearing either PC3 or DU145 xenografts. No significant changes in c-Myc concentrations were observed in the tumors of the mice following treatment with 10058-F4. This observation is in keeping with previous studies performed in vitro showing that, while inactivated by virtue of its dissociation from Max, c-Myc levels do not change appreciably (10,11).

Although these studies do not show efficacy for 10058-F4, this is not terribly surprising given the relatively low potency of the compound and its now determined short half-life. Nonetheless, they provide valuable information as to how studies with more potent compounds (see below) are best conducted in the future.

***Task 3*: To determine whether any of the compounds can be utilized in combination as a means of reducing toxicity and potentiating the antineoplastic effect.**

Our preliminary studies failed to show synergy between two structurally unrelated parental compounds such as 10058-F4 and 10074-A4 (not shown). This likely reflects the fact that the binding of these small molecules to their respective sites on the c-Myc bHLH-ZIP domain causes only local perturbations in peptide structure that fail to affect the other compound's binding at its cognate site. In contrast, there are several reasons why two linked Myc compounds that bind to such distinct sites might prove superior to the individual, unlinked compounds. First, given the proper spacing between the compounds, the binding of one to its cognate sites should increase the local concentration of the second compound at its own binding site, thus improving the probability of binding and allowing the compounds to be used at lower concentrations. In this sense, binding of a compound to its site indirectly affects subsequent binding of the second

compound at its site. Second, linking might reduce or eliminate non-productive interactions of the compounds with one another via hydrogen bonding, van der Waals, or hydrophobic interactions. Third, linked compounds might exhibit fewer non-specific or "off target" effects. Finally, there is experimental precedent for the use of such compounds (14,15). The strategy of multivalent binding (multiple ligands binding simultaneously to a single entity) is also ubiquitous in nature and has been exploited in multiple synthetic systems (14,16). The argument for linking Myc inhibitors specifically is bolstered by the relatively small size of some inhibitors: 10058-F4 and 10074-G5 are only 249 and 332 Da, respectively, a size which may still be considered a fragment for the purpose of fragment-based screening. The linkage of discreet fragments has been used to successfully inhibit a variety of protein targets (14).

Despite the appeal and theoretically sound basis of the linking approaches described above, the major question is whether in fact these approaches can be successful. Thus, we have confirmed the feasibility of the linkage strategy via the synthesis of a single linked compound as a proof of principle. The compound, termed "Link1", is composed of carboxylate derivatives of 10074-G5 and 10058-F4 linked via amide bond formation with pentane diamine (Fig 2a). Initial SAR and NMR models indicated that the modifications should not grossly interfere with binding (not shown). Link1 retained the fluorescence properties of each parental compounds. The direct binding affinity of Link1 for $Myc_{353-437}$ as determined by fluorescence polarization (K_D = 24±4 nM) was 100-fold better than the individual parental compounds (Fig. 2b). This enhanced affinity was also reflected in Link1's ability to compete with Max for binding of c-Myc (Fig. 2c). Link1 thus demonstrates the potential of one linkage strategy to drive the affinity of even non-optimized structures into the nanomolar range. The flexibility of the disordered protein target may serve as an advantage here, eliminating the need to match a rigid, specific spacing between the two binding sites on Myc. This compound is currently undergoing testing in cell-based assays.

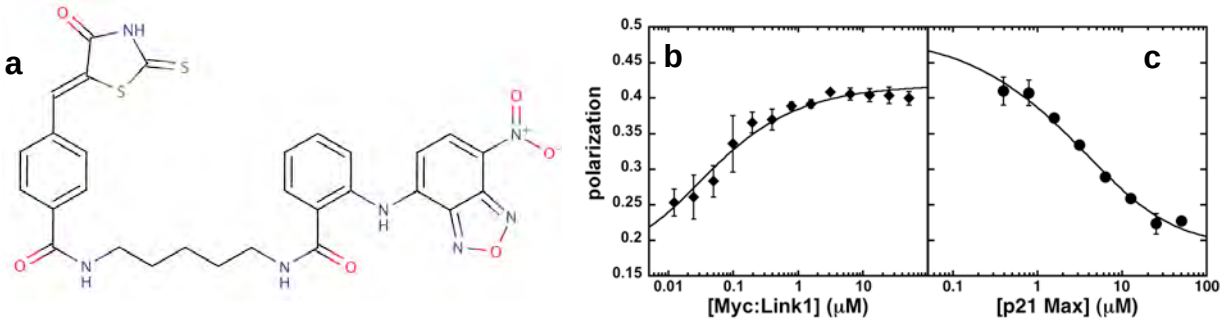


Figure 2. A) Structure of Link1 B) Fluorescence polarization of Link1 titrated at equimolar concentration with $Myc_{353-437}$ C) Fluorescence polarization of Link1 in complex with $Myc_{353-437}$ at 500 nM each with increasing concentration of Max competitor. A ten-fold excess of Max is required to reach 50% competition. Each graph represents the average of three trials.

Task 4: To employ computerized "data mining" techniques to identify the structural properties of these compounds which impart their effectiveness.

The major problem with all Myc compounds described here and elsewhere is their relatively low potencies, with significant tumor cell inhibition being attained only at 50-100 μM (10). For this reason, their clinical application is limited. Because compound 10058-F4 has the simplest structure (Fig. 3), we used it to determine whether, in the absence of any 3D structural information, we could identify more potent analogs.

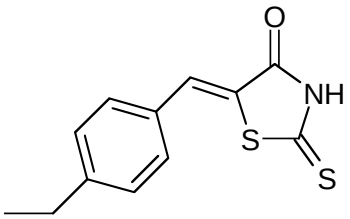


Fig. 3. The structure of 10058-F4, one of the original c-Myc parental compounds.

We therefore conducted an *in silico* screen of the 5040 member ChemDiversity (Chem Div, San Diego, CA) library for structures that share the same 5-member rhodanine ring as the parental compound but that contain variations of the 6-member ring. This search yielded ten so-called "2nd generation" compounds. We also synthesized a library of 38 additional 10058-F4 analogs with alterations in only the 6-member ring (11) All were tested for their ability to inhibit the growth of human HL60 promyelocytic leukemia cells, which expresses high levels of c-Myc as a result of gene amplification. Because this assay is biologically based, it served as an easy, rapid, and accurate means of eliminating inactive agents. Compounds scoring as "hits" were subjected to additional analyses for specificity, including the ability to disrupt c-Myc-Max dimers in HL60 cells, and to prevent the association of recombinant c-Myc-Max and DNA binding *in vitro* using electrophoretic mobility shift assays (EMSAs). The detailed results of these screens can be found in Ref. 11 and are only summarized here.

We identified several 6-member ring analogs that were at least as potent as 10058-F4 at inhibiting HL60 growth. One analog, 27RH, was approximately twice as potent as 10058-F4 (IC50 23 μM vs. 51 μM) whereas a second, 28RH, was only marginally better (IC50 36 μM). Although profoundly growth inhibited, the cells in all cases remained >80% viable after 4-5 days as determined by trypan blue staining (not shown). Thus, alterations of the six-member ring of the 10058-F4 index compound occasionally lead to significant but modest improvements of in vivo activity.

An additional *in silico* screen of a 500,000 member library (ChemBridge San Diego, CA) was then conducted to identify 10058-F4 analogs modified only in the five-member rhodanine ring. Eleven compounds were identified, four additional ones were synthesized (11) and all were then tested in HL60 cells. Numerous compounds retained activity and four (1RH-S-Me, #015, #474, and #764) were significantly better than 10058-F4 (IC50's=4.6-18 μM) (11).

Active 10058-F4 5- and 6-member ring analogs were next tested for their ability to inhibit c-Myc-Max dimerization in HL60 cells and to prevent or reverse heterodimerization and DNA binding by recombinant c-Myc and Max *in vitro*. We confirmed this to be the case and demonstrated a good, albeit inexact correlation between these assays and the inhibition of HL60 growth (11).

The foregoing results indicated that certain "random" modifications of 10058-F4's component rings lead to improved activity by as much as ca. 6-8-fold, particularly when the 5-member ring was modified. In order to determine whether the idealized ring structures from these compounds could be combined additively, we next synthesized and tested a group of 17 compounds containing select combinations of optimized 5- and 6- member rings (11). The choice of each starting ring structure was based on a combination of the results of HL60 and EMSA assays described above as well as upon the ease of synthesis and yield of the final compound. Only two compounds, 12RH-NCN-1 and 28RH-NCN-1, demonstrated activities comparable to, but not better than that of 10058-F4 in HL60 cells. From these and previous studies, we conclude that the greatest improvements in efficacy resulted from changes of the rhodanine ring of 10058-F4. We have discussed the reasons why the combination of optimized 5 and 6 member rings together fail

to provide significant improvement in activity compared to what is seen with a single modified ring (11).

It was clear from the above studies that non-structure-based modifications of the chemical structure of 10058-F4 could occasionally lead to the identification of compounds with significantly improved activities. However, such studies clearly suffered from the lack of a 3D structure of compounds in association with their cognate binding sites. To this end, we have recently completed 3D NMR models of these sites in collaboration with Dr. Steven J. Metallo (Dept. of Chemistry, Georgetown University). Although the details of how these models were derived are complex and are not provided here, Fig. 4 does show the summary of these results, which have been submitted for publication (17).

Taken together, these new results provide us with a structural rationale for the design of a whole new generation of low molecular weight compounds whose design will utilize the basic structures of 10058-F4, 10074-G5, and 10074-A4. Our success with linking two non-optimized low molecular weight compounds and observing a >100-fold increase in affinities for the c-Myc bHLH-ZIP peptide provides reason to be optimistic that even greater increases in efficacies will be achieved when the individual linked entities are based on optimized structures derived from the NMR models shown here.

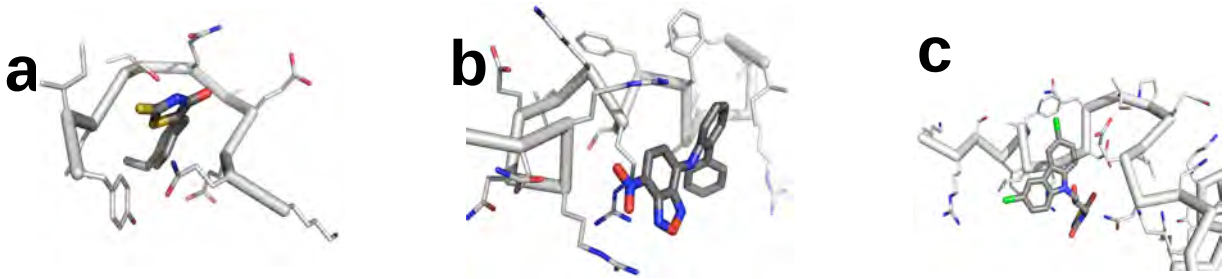


Fig. 4. (a). NMR-derived model of the complex between 10058-F4 and the synthetic peptide encompassing its binding site (amino acids 402-412). (b). Structural model of the complex between 10074-G5 and the synthetic peptide encompassing its binding site (amino acids 363-381). (c). Structural model of the complex between 10074-A4 and the recombinant peptide encompassing its binding site (amino acids 370-409).

SUMMARY

Seven low molecular weight compounds were initially identified that were capable of disrupting/preventing the association between c-Myc and its obligate heterodimerization partner Max. As a result of this DOD grant, we have been able to show that three of these seven molecules bind to distinct sites within the c-Myc bHLH-ZIP domain but do not bind to Max. These findings have enabled us to obtain NMR structures of each of these compounds in association with its cognate binding site on the c-Myc monomer. Preliminary studies performed with one of the compounds (10058-F4) prior to obtaining its NMR structure indicated that a number of “randomly” generated analogs could be obtained that possessed significantly higher activity than the parental molecule. These observations provide reason to be optimistic that new analogs for all three compounds, now obtained rationally based on their NMR structures, should be even more potent and thus excellent pre-clinical candidates. To this end, preliminary studies have shown that linking two of the parental compounds, each of which binds to distinct sites, results in ca. 100-fold increases in c-Myc binding. The develop of in vivo testing methodologies during this funding period has also set the stage for the rapid assessment of the pharmacologic and therapeutic assessments of these novel compounds.

KEY RESEARCH ACCOMPLISHMENTS STEMMING FROM THIS AWARD

1. Determination that all c-Myc compounds bind to monomeric c-Myc.
2. Identification of the precise binding sites for three of the original seven c-Myc compounds, 10058-F4, 10074-A4, and 10074-G5.
3. Determination that the remaining four compounds bind to one of the above three sites.
4. Development of quantitative fluorescence polarization- and circular dichroism-based assays for c-Myc compound binding.
5. Identification of more potent analogs based on random chemical modifications of parent compound 10058-F4.
6. Solving the 3D NMR structures of 10058-F4, 10074-A4, and 10074-G5 in association with their peptide binding sites.
7. Demonstrating that linking two unrelated compounds provides for a ca. 100-fold increase in potency
8. Determination of the pharmacologic properties of 10058-F4.

REPORTABLE OUTCOMES/PUBLICATIONS STEMMING FROM THIS AWARD

Wang H, Hammoudeh DI, Follis AV, Reese BE, Lazo JS, Metallo SJ, Prochownik EV. Improved low molecular weight Myc-Max inhibitors. Mol Cancer Ther. 2007. 6: 2399-408. See attached

Guo, G., Parise, R. A., Joseph, E., Egorin, M.l J, Lazo, J. S. , Prochownik, E. V. and Eiseman, J. L. Efficacy, pharmacokinetics, tissue distribution, and metabolism of the Myc-Max disruptor, 10058-F4 [Z,E]-5-[4-ethylbenzylidine]-2-thioxothiazolidin-4-one, in mice. Cancer Chemother. and Pharmacol. (in press). See attached

Viacava Follis A, Hammoudeh D, Wang H, Prochownik EV, Metallo SJ. Binding of small-moleculae inhibitors to local sequence sites on the intrinsically disordered c-Myc oncoprotein. Submitted for publication.

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